MEASUREMENT OF COLLAGENASE ACTIVITY IN THE TANNERY AND IN THE LABORATORY I. THE ASSAY MEDIUM*†

by

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ABSTRACT

A commercially available acidic solution of pepsin-treated porcine skin collagen designed for cosmetic use was shown to be suitable as a substrate for viscometric assay of collagenase activity when adjusted to near neutrality and diluted with the proper buffer to a collagen concentration of 1.5 to 4.5 g/L. The buffer contains tris(hydroxymethylamino) methane (Tris), citrate, and calcium (Ca^{+2}). The pH of the assay solution is 6.8. The assay solution can be supplemented with NaCl up to a concentration of at least 0.4M and the pH can be raised to at least 8.5. Pepsin treatment of calfskin collagen yields a product whose viscometric behavior in the presence of collagenase is virtually identical to that of the commercial porcine product.

INTRODUCTION

Procedures are needed for rapid estimation of collagenase activity in the tannery and in the leather research laboratory. Such procedures would serve to measure the extent to which various microorganisms, including halophiles, produce collagenases, and to evaluate bate and hides for contamination by these enzymes. Collagenases are important to the leather processor because the substrate whose degradation they initiate is the major structural component of leather.

Our strategy was to develop a procedure for the leather research laboratory which would then be simplified to provide a procedure for the tannery. The assay medium (i.e., the substrate and the buffer components) is, of course, the same for the two procedures. The composition of an assay medium suitable for our work, as well as for any viscometric measurement of collagenase activity using collagen as substrate, is the subject of this paper. The apparatus we have developed to implement rapid measurement of collagenase activity using this assay medium will be presented in a separate communication.

Synthetic substrates are available for assay of "collagenase" activity. However, interpretation of results obtained with these peptides is ambiguous. Enzymes have been found which hydrolyze collagen but do not hydrolyze one or more of the synthetic substrates, and others which do not hydrolyze the protein but do hydrolyze one or more of the peptide substrates[1,2]. Since our interest is specifically in the hydrolysis of collagen, we opted to use the protein itself as substrate. Viscometry was selected as the means of measuring this hydrolysis because of it sensitivity, precision, and reliability[3,4].

MATERIALS AND METHODS

Materials and Solutions

Viscosity measurements were made with size 100 and size 200 Cannon-Fenske Routine viscometers purchased from the Cannon Instrument Co., State College, PA. The

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† Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

temperature was controlled at 19.93 ± 0.02°C by a model M-1 Constant Temperature Bath from the same company. Cacitris Buffer contained 0.25M Tris, 0.12M citrate (Na), and 10.0 mM $CaCl_2$, and had a pH of 6.80 ± 0.05 at 25°C. Pepsin-treated porcine collagen, produced by Pentapharm Ltd., Basel, Switzerland, was purchased from Centerchem, Inc., Stamford, CT, as Natural Soluble Collagen (Dermacol), a solution with the following composition: 1.0 ± 0.1% collagen, 3.3% sodium citrate, 0.19% sodium benzoate, pH 3.6. The pH was adjusted to 6.80 ± 0.05 by the addition of 2M Tris (base), and 2M $CaCl_2$ was added to a concentration of 8.8 mM; the solution was then diluted with Cacitris buffer to the desired substrate (pepsin-treated porcine collagen) concentration, either 5.03 g/l or 1.76 g/L. Acid soluble calfskin collagen (classification type I) prepared according to Gallop and Seifter[5] was purchased from Sigma Chemical Co., St. Louis, MO. The collagenase, obtained from Clostridium histolyticum, was Sigma type IA, with a specific activity of 320 collagen digestion units/mg solid. It was dissolved in Cacitris buffer to a concentration 8 times the concentration desired in the assay medium.

Preparation of Pepsin-Treated Calfskin Collagen

Five hundred mg acid-soluble calfskin collagen were added to 20 mL 0.50M acetic acid containing 15 mg pepsin (cf ref. 6). The solution was stirred for 4 days. 1.17 g NaCl was added slowly while stirring[7]. The resulting suspension was centrifuged in a Sorvall SS-34 rotor at 10,000 rpm for 30 minutes, and the precipitate was dissolved in 25 mL Cacitris buffer. Fifteen mL of this solution were diluted to 60 mL with Cacitris buffer, yielding a substrate (pepsin-treated calfskin collagen) concentration of 2.61 g/L. All solutions and equipment were at 4°C[6,7].

Measurement of Viscosity

The viscometer and the substrate and enzyme solutions were preequilibrated to 19.93 ± 0.02°C in the constant temperature bath. A 7.00 mL aliquot of the substrate solution was pipetted into the viscometer, followed by 1.00 mL of the enzyme solution. After mixing, outflow times were measured repeatedly during an incubation period of approximately one hour.

Results and Discussion

Assay of enzyme activity by solution viscometry requires careful choice of all components of the assay medium. In particular, for the viscometric assay of collagenase activity using collagen as substrate, a form of collagen which is soluble near neutrality is necessary. Most collagen preparations are at least partially insoluble in commonly used buffer systems with near-neutral pH's, where the collagenases are active.

Treatment of native collagen with proteolytic enzymes, such as pepsin or trypsin, results in hydrolysis of peptide bonds in the relatively small telopeptide region of the molecule; the triple helical region, which includes the bulk of the molecule, is not directly affected. However, because such treatment creates numerous $-COO^-$ and $-NH_3^+$ groups with access to the solvent, solubility is increased. Since pepsin-treated porcine skin collagen with minimal crosslinking is commercially available (in solution form) at relatively modest cost, it was examined for its suitability as substrate for assay of collagenase activity. For comparison, a preparation of pepsin-treated acid soluble calfskin collagen was made in our laboratory and similarly examined.

Gallop and Seifter[3,4] carried out now-classical investigations into the viscometric assay of collagenase activity. They used a fish collagen as substrate, and a solvent 0.5M in $CaCl_2$ to render it soluble. With the expectation that our substrate was of higher solubility than theirs, we decided to lower the Ca^{+2} concentration to 10 mM, in keeping with levels commonly used for enzymes requiring Ca^{2+}. We found that the pepsin-treated collagen is soluble at this low level of Ca^{+2} in the presence of 0.25M Tris, 0.12M citrate, and a pH of 6.8 to 8.5 (at 25°C) (Compare "Cacitris buffer" in Materials and Methods section).

Figure 1a shows the result of an experiment on the hydrolysis of the pepsin-treated porcine collagen by five levels of collagenase in Cacitris buffer. The substrate concentration in the assay medium was 4.4 g/L. The enzyme concentration varied from 0.5 (run 1) to 2.5 g/L (run 5) in increments of 0.5. The viscometer was size 200. The outflow time of the collagen-collagenase solution is t; t_s is the outflow time for the solvent. $\bar{T}$ (T bar) is the incubation time halfway through the outflow period. More specifically, letting T_1 be the time (since addition of the enzyme to the substrate) when the meniscus of the incubate passes the upper mark of the viscometer and T_2 the time when it passes the lower mark, $t = T_2 - T_1$ and $\bar{T} = T_1 + (T_2 - T_1)/2 = (T_1 + T_2)/2$. The equation for each of the lines plotted in Figure 1a can be written as

$$t = t_s + \Delta t e^{-m\bar{T}} \qquad (1)$$

where Δt is a constant ($\Delta t = t_0 - t_s$, where t_0 is the outflow time at $\bar{T} = 0$, i.e., before the collagenase has had a chance

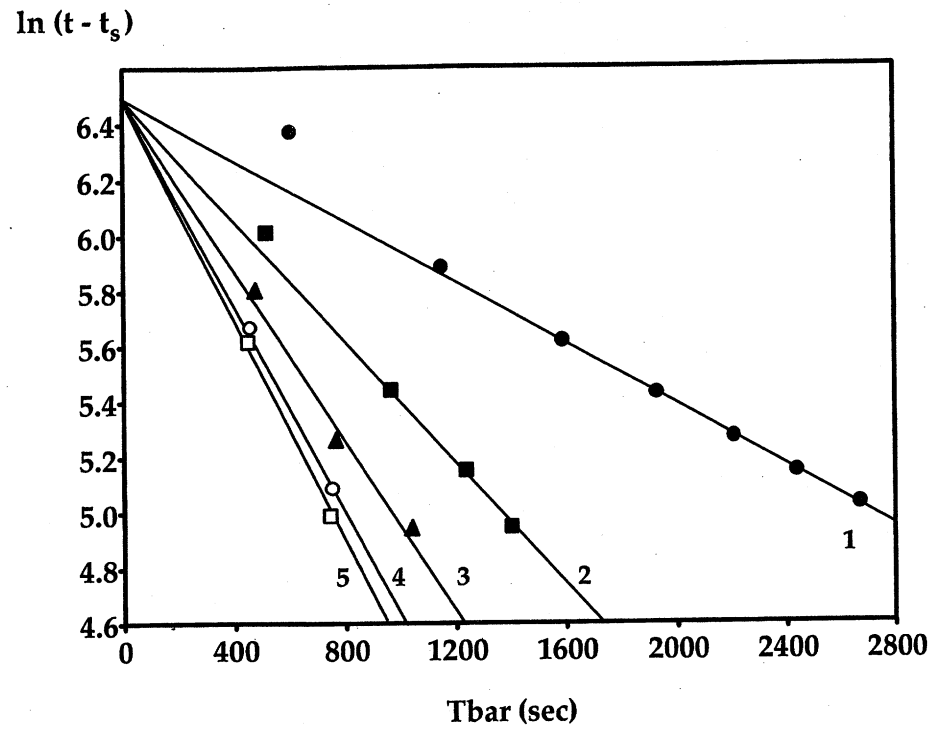

FIGURE 1a. — Plot of the natural logarithm of the adjusted outflow time ($t-t_s$) vs. mean incubation time ($\bar{T}$) for the hydrolysis of pepsin-treated porcine skin collagen by Clostridium histolyticum collagenase. The initial Collagen concentration was 4.40 g/L. The enzyme concentration varied from 0.5 (run 1) to 2.5 g/L (run 5) in increments of 0.5. t is the outflow time of the sample containing collagen and collagenase, t_s is the outflow time for the solvent.

to act; in Figure 1a, $\ln \Delta t = 6.52$.) The rate constant m is a parameter which varies with enzyme concentration. Since the specific viscosity $\eta_{sp} = (t-t_s)/t_s$, $\ln (t-t_s)$ differs from $\ln \eta_{sp}$ only by a constant, $\ln t_s$. $\ln \eta_{sp}$ is the quantity plotted by Gallop and Seifter[3,4] and others in their analyses of viscometric data.

When the magnitude of the slope (m) of each of the lines in Figure 1a is plotted against the enzyme concentration (c_E), Figure 1b is obtained. The slope data are fitted very well by a straight line through the origin for $c_E \leq 1.5$ g/L (480. units/mL). This agrees with the result obtained by Gallop and Seifter[3,4] with fish collagen.

The substrate concentration can be reduced if a viscometer having a capillary of narrower bore is used. Figure 2 shows the results of a series of runs with a substrate (pepsin-treated porcine collagen) concentration in the assay medium of 1.54 g/L, approximately one-third of that in the experiment of Figure 1. The viscometer was size 100, which has an internal diameter (id) of ca. 0.63 mm; the id of the #200 viscometer (Figure 1) is ca. 1.01 mm. [It should be recalled that, according to Poiseuille's law[8], resistance to flow varies inversely with the fourth power of the capillary diameter.]

The collagenase was of the same type as in the previous experiment (Figure 1). The collagenase concentration in the assay medium varied from 0 to 0.125 g/L (in increments of 0.025 g/L). To hydrolyze fragments of collagen produced by the action of collagenase, trypsin was added to a concentration of 50 mg/L. The data have been normalized by dividing every outflow time t by $t_0 = 6.46$ (min), the outflow time for $c_E = 0$.

Unlike the data of the previous experiment, those of this experiment (shown in Figure 2 by circles, squares, triangles, and x's) could not be fitted well by functions with a single exponential decay term (equation 1). However, the data of every run are fitted quite well by a function with two exponential decay terms (see curves of Figure 2) as found by von Hippel et al[9] in their work with fish collagen. The equation for double exponential decay (not normalized) can be written as

$$t = t_\infty + \Delta t_1 e^{-k_1 \bar{T}} + \Delta t_2 e^{-k_2 \bar{T}} \qquad (2)$$

where t_∞ is the outflow time after hydrolysis by collagenase is complete. The equation describes the variation of outflow time t (which is proportional to viscosity) with mean incubation time $\bar{T}$ resulting from two simultaneous

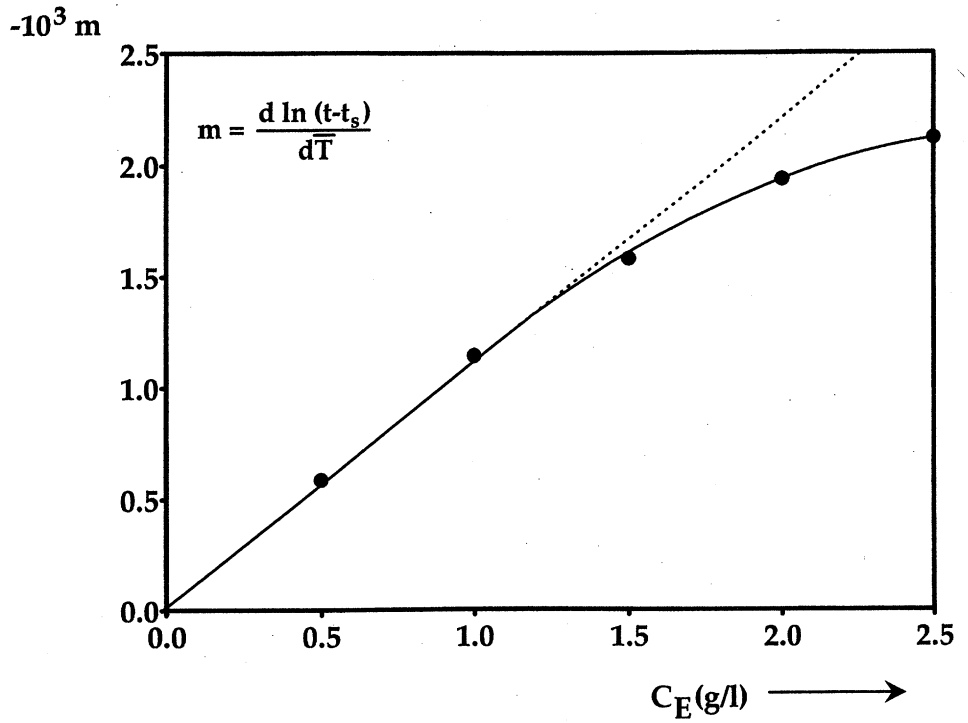

FIGURE 1b. — Dependence of the magnitude of the slope of 1n ($t-t_s$) vs. $\bar{T}$ (Figure 1a) on the enzyme concentration (c_E).

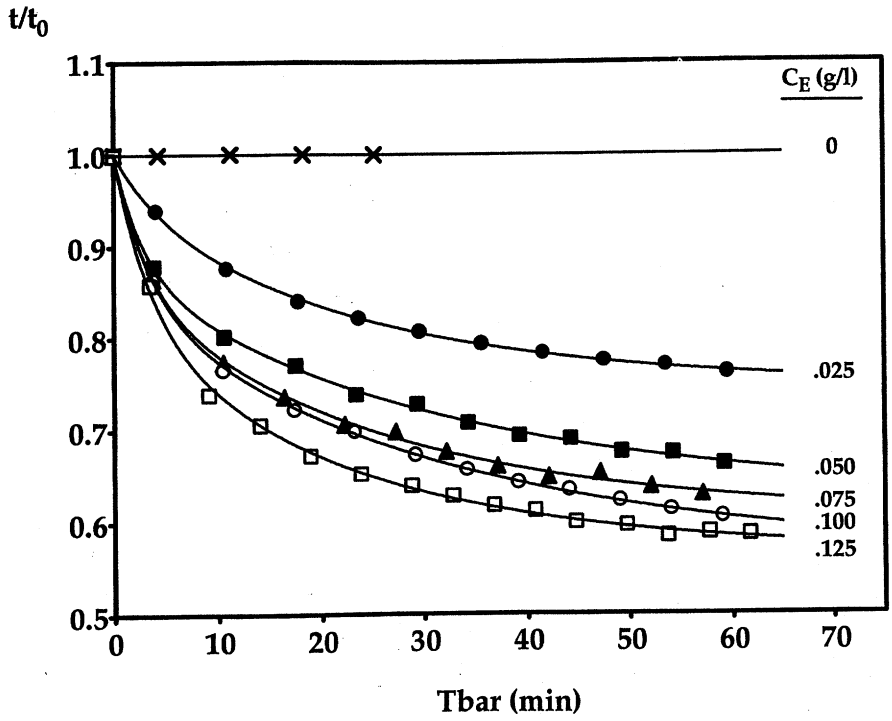

FIGURE 2. — Dependence of the normalized outflow time ($t-t_0$) on the mean incubation time for the hydrolysis of pepsin-treated porcine skin collagen by Clostridium histolyticum collagenase at various concentrations of the enzyme. The initial collagen concentration was 1.54 g/L. t_0 is the outflow time in the absence of collagenase (= t in the presence of collagenase at T = 0).

reactions having rate constants k_1 and k_2. Letting $\Delta t = t_0 - t_\infty$, the drop in outflow time resulting from complete hydrolysis by collagenase, $\Delta t = \Delta t_1 + \Delta t_2$, where Δt_1 is the part of Δt attributable to reaction 1 and Δt_2 is the part of Δt attributable to reaction 2. $\Delta t_1 e^{-k_1 \bar{T}}$ is thus the reaction 1-sensitive contribution to the outflow time t at time $\bar{T}$ and $\Delta t_2 e^{-k_2 \bar{T}}$ is the reaction 2-sensitive contribution.

The reason that the data of Figure 1a can be fitted with an equation with only one exponential decay term (equation 1), while those of Figure 2 (and Figure 3a below) require an equation (equation 2) with two such terms, is as follows. All viscosity data of the type dealt with here are described by equation 2. However, under some conditions, the middle term on the right side of equation 2 is much smaller than the last term and can be disregarded. Δt_1 and Δt_2 (equation 2) are known to be of comparable magnitude, and $k_1 \gg k_2$ (Figures 3b and 3c). k_1 and k_2 both increase with collagenase concentration (c_E,). Therefore, for relatively long incubation times ($\bar{T}$) and high c_E, $\Delta t_1 e^{-k_1 \bar{T}} \ll \Delta t_2 e^{-k_2 \bar{T}}$, and equation 2 reduces to equation 1. The collagenase concentrations for the experiment described by Figure 1a were much larger than those for Figure 2 (and Figure 3a), and for the earliest data point in the former case $\bar{T}>7$ minutes. In keeping with this interpretation, the earliest data points for the two runs (runs 1 and 2 in Figure 1a) with the lowest enzyme concentrations deviate positively from the lines drawn through the remaining data points.

In the absence of collagenase (upper curve of Figure 2) the viscosity was invariant with time. Since trypsin was present, this result shows that the substrate behaves as a native collagen, i.e., it is not affected by general proteolytic activity.

The viscometric behavior of pepsin-treated collagen from calfskin (see Materials and Methods) as a substrate for collagenase was also examined. The conditions were the same as those described above (in connection with Figure 2) for the porcine substrate, except as follows: the substrate concentration $c_s = 2.28$ g/L, $t_0 = 8.71$ min, and c_E varied from 0 to 0.25 g/L in increments of 0.05. In analyzing the data by curve fitting (equation 2), all curves (except that for $c_E = 0$) were required to have a common asymptote, $t_\infty/t_0 = 0.334$. A plot of the normalized outflow time t/t_0 against the mean incubation time $\bar{T}$ is shown in Figure 3a, together with the double exponential decay curves fitted to the data. The fit is clearly good for every run. The

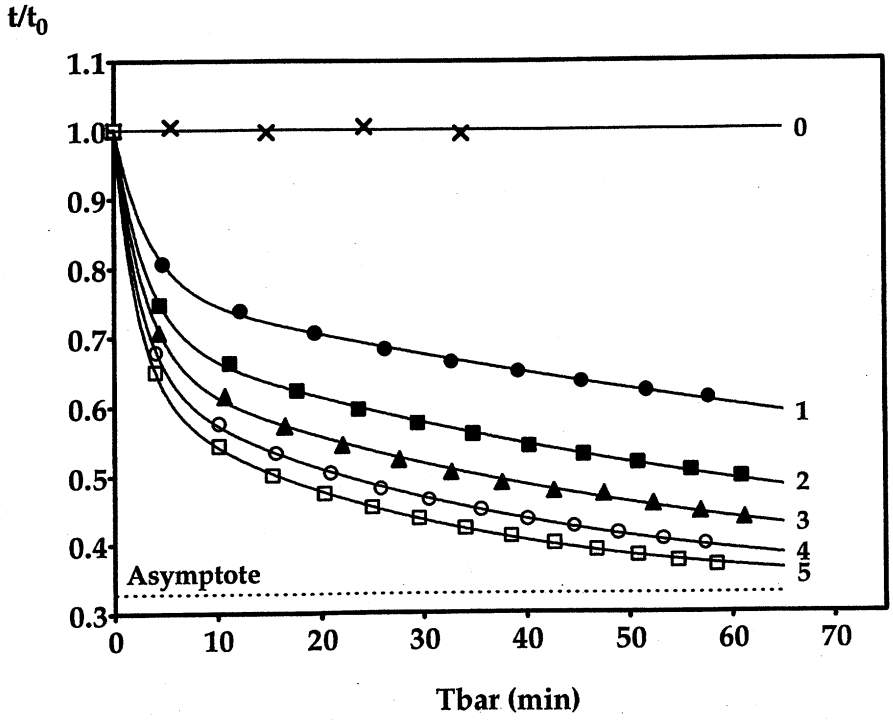

FIGURE 3a. — Dependence of the normalized outflow time on the mean incubation time for the hydrolysis of pepsin-treated acid-soluble calfskin collagen by Clostridium histolyticum collagenase at various concentrations of the enzyme. The initial collagen concentration was 2.28 g/L. The enzyme concentration varied from 0 (upper curve) to 0.25 g/L (run 5) in increments of 0.05.

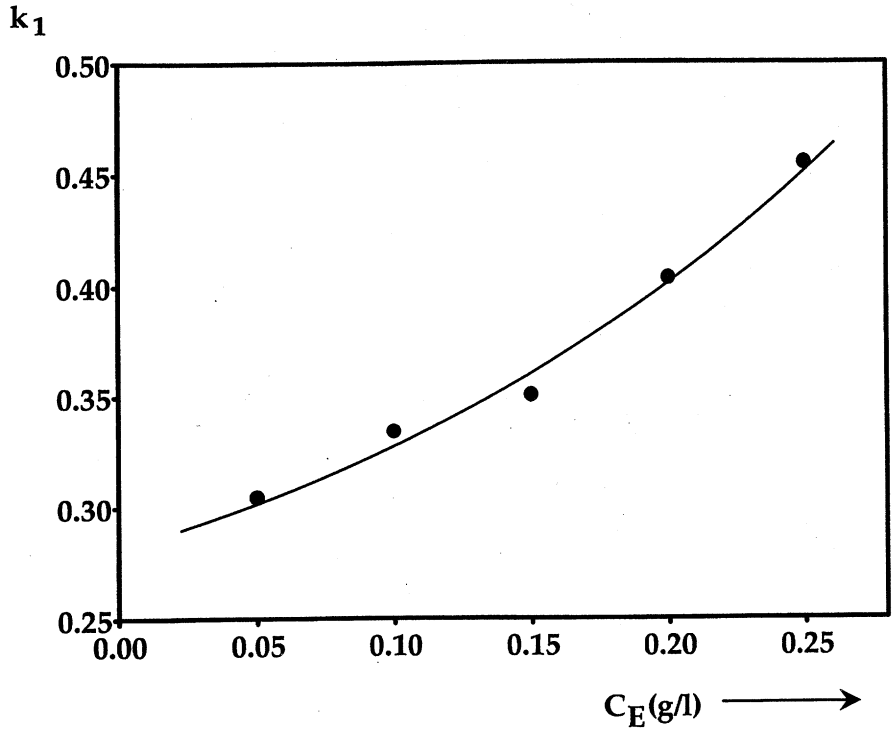

FIGURE 3b. — Apparent dependence of the decay constant for the fast reaction (k_1) on the enzyme concentration. k_1 was obtained from the curves in Fig. 3a.

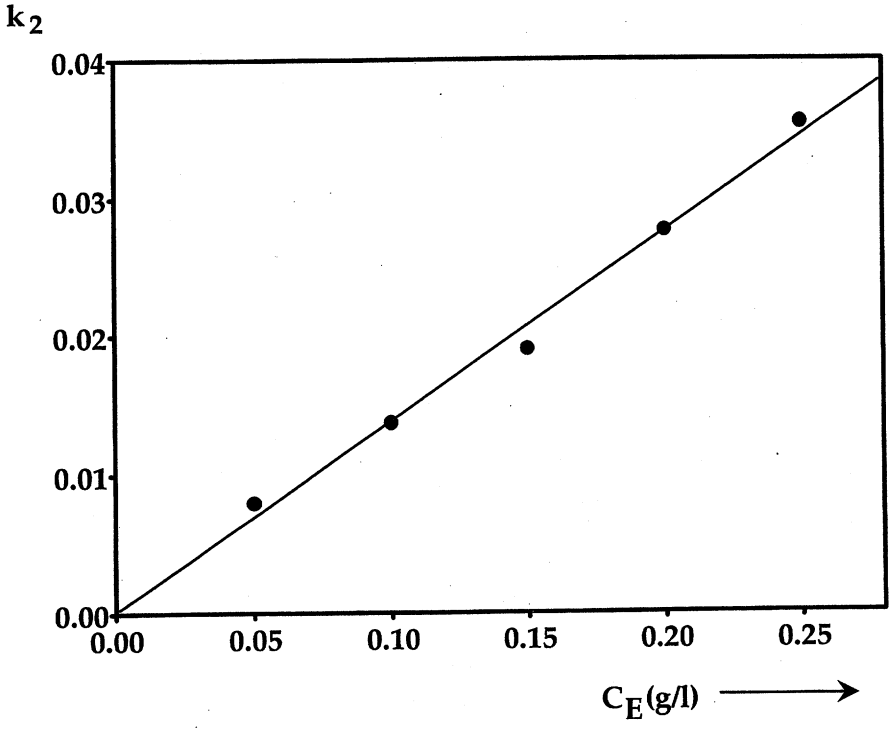

FIGURE 3c. — The decay constant for the slow reaction (k_2) as a function of the collagenase concentration. k_2 was obtained from the curves in Fig. 3a.

similarity of the behavior of the porcine skin (Figure 2) and calfskin (Figure 3a) collagens is obvious.

When the decay constants k_1 and k_2 for the curves in Figure 3a are plotted against enzyme concentration c_E, the plots shown in Figures 3b and 3c are obtained. k_2 (Figure 3c) is thus proportional to enzyme concentration; k_1 (Figure 3b) is not. The lack of proportionality for k_1 is probably the result of the slowness of the Cannon-Fenske measurement in relation to the rapidity of reaction 1, especially early in the incubation period, when the solution viscosity is still high. Thus, for the first data point obtained for c_E= 0.10 g/L, $\bar{T}$ = 4.37 min and t = 6.51 min. For this run $k_1 = 0.334\ min^{-1}$. Therefore $e^{-k_1\bar{T}} = 0.232$. Thus, the fast reaction, reaction 1, is already 76.8% complete after 4.37 min incubation, and the outflow time then is 6.51 min. The frequency of measurement possible with the Cannon-Fenske viscometer is thus not sufficient to provide accurate data for the fast reaction. The data are entirely adequate for the slow reaction.

While the experiments discussed above demonstrate that pepsin-treated collagen (in Cacitris buffer) is a suitable substrate for viscometric assay of collagenase activity, they also indicate the unsuitability, for routine work, of measuring the viscosity with an Ostwald-type (Cannon-Fenske) viscometer. The procedure is very labor intensive, and data for early incubation times, which are needed for a rapid assay procedure, cannot be obtained. The development of a relatively simple apparatus providing viscosity data very early in the incubation will be described in a forthcoming paper. The impetus for this approach derives from equation 2. Differentiating this equation, we obtain

$$\frac{dt}{d\bar{T}} = -k_1\Delta t_1 e^{-k_1\bar{T}} - k_2\Delta t_2 e^{-k_2\bar{T}} \qquad (3)$$

At $\bar{T} = 0$

$$\left[\frac{dt}{d\bar{T}}\right]_{\bar{T}=0} = -k_1\Delta t_1 - k_2\Delta t_2. \qquad (4)$$

Assuming $k_1 = \lambda_1 c_E$ and $k_2 = \lambda_2 c_E$, where λ_1 and λ_2 are constants,

$$\left[\frac{dt}{d\bar{T}}\right]_{\bar{T}=0} = (-\lambda_1\Delta t_1 - \lambda_2\Delta t_2)\, c_E \qquad (5)$$

Therefore,

$$\left[\frac{dt}{d\bar{T}}\right]_{\bar{T}=0} = -kc_E \qquad (6)$$

where $k = \lambda_1\Delta t_1 + \lambda_2\Delta t_2$.

The initial rate of decrease of viscosity (or outflow time t) is thus proportional to the enzyme concentration. It is therefore unnecessary to collect data for an extended period of time, such as tens of minutes. An incubation period of 5 minutes is sufficient.

CONCLUSIONS

A commercially available pepsin-treated collagen from the skin of young pigs has been shown to be a suitable substrate for viscometric assay of collagenase activity. It is supplied as a solution; to use it for assay of collagenase, it is only necessary to adjust the pH and dilute it with the appropriate buffer. This removes an important disincentive to the use of collagen as substrate for the assay of collagenase activity, viz., the unavailability of a collagen requiring little or no treatment before use.

The viscometric behavior of the porcine product was shown to be very similar to that of pepsin-treated calfskin collagen, thus confirming the suitability of the commercial porcine product.

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DISCUSSION

Robert M. Good, Pfister & Vogel Leather Co.: Do I understand that one of the simplifying aspects of this is to conduct the measurement at room temperature or is temperature somehow controlled in a way that I was not made aware of?

Answer: Temperature control in a rapid experiment, say in a five minute experiment, is not nearly as critical as it is in a long term, say a one hour experiment, such as those that I showed before where you have to collect the data for an hour or a little more than an hour. On the short term experiments, you can easily get by if you have a room that doesn't change temperature too rapidly.

Don Boyce, Novo Nordisk Bioindustrials: Have you had a chance to correlate your viscometry results possibly with an osmometer or a freezing point depression apparatus? The latter is particularly good in a situation where you have a hydrolysis of an insoluble substrate which is being made soluble?

Answer: I haven't done anything like that.

Don Boyce: They take about two minutes per tube and are used in the dairy industry for quality control.

Answer: Do you think it would be capable of detecting something like this kind of a change in viscometer reading with the collagenase?

Don Boyce: You won't see a viscometry change, but you'll see a freezing point depression change. It's very sensitive and it's particularly good where you have an insoluble substrate being made soluble by the enzymatic action. Plus, you're not restricted by having to identify a capillary to detect the viscosity range of your experiment.

Juergen Christner, Rohm Tech, Inc.; I would like to know how the results of this exciting new method correlate with the practical results found on actual application of bating enzymes to hides in normal leather manufacture?

Answer: We haven't done any work yet on actual applications to hides. All of this is just the results of the methodology. We do have in mind to extend this type of an approach to bate analysis and drum liquor analysis, but then, of course, we'll have to change the substrate from collagen to something else. Gelatin in one possibility, casein is another possibility, but we would use the same viscosity technique to make those measurements. However, we haven't done any applications to a practical tanning situation yet.